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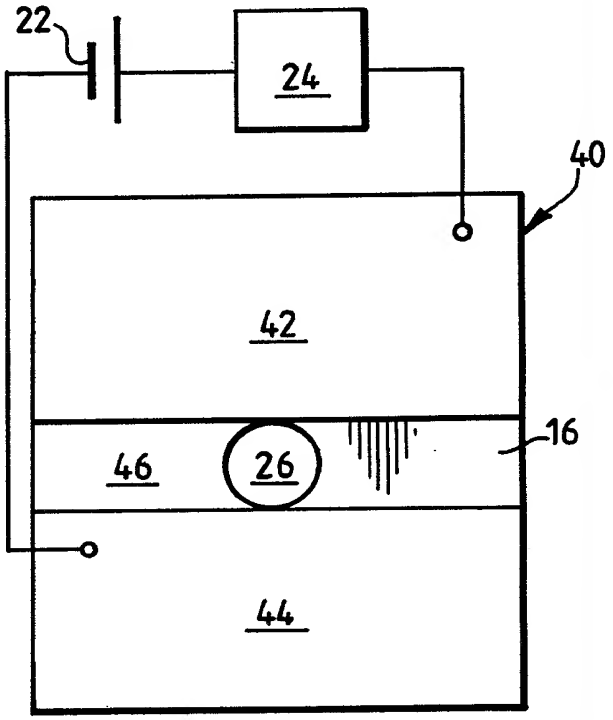
(57) Abstract

In a method and apparatus for examining samples comprising individual objects (28) of macromolecular or similar size, or smaller, an instrument element (14) comprises a substrate (16) overlaid with a thin film layer (18) of a material that is electrically conductive and/or at last partly optically opaque. A discontinuity comprising an aperture (26) or an asperity is formed in or on the element (14) in a known location, and the sample (28) is brought to this discontinuity so that it is not necessary to search for the sample by scanning. Energy (e.g. electrical energy or light) is applied to the element (14) and, with the latter and the sample in intimate association, e.g. with the sample inside the aperture (26), changes in the radiation from the sample site resulting from the presence of the sample are detected.



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EXAMINATION OF OBJECTS OF MACROMOLECULAR SIZE

This invention relates to methods and apparatus for examining individual objects the size of which is of the general order of magnitude of macromolecules and their aggregates, or smaller. These objects may consist of particles, or of other materials or structures of generally similar dimensions. Where they are particles, they may in fact actually be macromolecules, for example enzymes or other proteins, or biological macromolecules or other larger structures such as viruses. The field covered by the invention does not however exclude the examination of particles of generally sub-micron size. Objects for examination, or being examined, will sometimes herein be called "samples", for convenience.

Current techniques, such as scanning tunnelling microscopy, atomic force microscopy and scanning near field optical microscopy, are already well known, and have considerably lowered the limit of molecular resolution that is now possible. In all these techniques, the image is built up from a scanned signal which is generated by interaction of the structure of the sample particle with a probe tip having dimensions comparable with that of the sample.

In scanning tunnelling microscopy (STM) an ultra-fine, chemically etched electrode is brought very close to the sample, so that electrons can pass by quantum tunnelling across the free space between the electrode tip, or part thereof, and the surface of the sample. The samples are mounted on a conductive substrate, and the probe is scanned across the substrate in order to find a sample for examination. Piezoelectric transducers are used to control the movement of the probe. One disadvantage of

STM is that the sample must be sufficiently electrically conductive, and with many types of particle, particularly biological particles, this requires pretreatment of the sample macromolecule in order to improve its conductivity. This has been achieved by using metal shadowing or doping with conductive salt ions; such treatment can however alter the characteristics of the sample, so that in some cases it becomes self-defeating.

Another, somewhat similar, device is the scanning capacitance microscope, which measures the capacitive changes associated with sample structures but on a much lower resolution. It can operate to the same order of magnitude of sample size as conventional optical microscopy, or larger.

In atomic force microscopy (AFM), an ultra-fine, very lightly spring loaded probe tip is scanned across an area containing a sample, and then across the sample itself. The movement of the tip as it is deflected by repulsion between the Van der Waals forces between the tip and the sample is monitored so as to generate a topographical image of the surface of the sample. A major disadvantage of AFM is that with most biological macromolecular structures, particularly in solution, the forces generated by the probe tip will tend to damage or destroy the sample. The AFM technique is therefore somewhat limited in scope, and, as is also generally true of STM, it is really only suitable for use where the sample can be shadowed, i.e. coated, with metal.

Electromagnetic radiation can be greatly amplified when interacting with strongly curved parts of a surface due to quasi-static concentration of field lines (the so-called "lightning rod" effect). This effect is responsible for the development of surface-enhanced Raman

scattering or roughness-induced electrical breakdown. It is also responsible for the strong elastic light scattering associated with microscopic holes in a film (apertures) or protrusions (asperities) from a surface, a process closely related to Mie scattering from small spheres which act as "short optical antennae" (SOA). The degree of scattering from these SOA is related to their size (curvature) and dielectric properties.

The method employed in a scanning near field optical microscope (SNOM) is a variant on the STM technique, and is essentially optical in character. The SNOM includes a component which serves as an optical source of suitably small dimensions and which performs an active function in the examination process. Such a component will be found in what will be generally called an "instrument element" in this description. In the SNOM, the instrument element can comprise a substrate of a suitable wave guide material, generally flat and having a very thin layer of gold deposited on one surface. The gold is so applied that it has small apertures or asperities (generally no larger than 100 nm), which act as centres for light scattering. In other words, the apertures or asperities act as short optical antennae. The effect of this scattering is that some radiation leaks from the apertures or asperities in the gold film, the intensity of the scattered radiation being dependent on the size of the aperture and on the dielectric properties of the materials. The scattered light emanating from the apertures or asperities is easy to detect using conventional optics. The SNOM carries out optical topographical imaging of samples by monitoring the changes in intensity of the light scattered from the aperture or asperity as the sample is brought into very close proximity to the radiating near field emanating

from the aperture or asperity. In the near field region, the electric field of the radiation is severely damped by the approaching sample, and this sensitivity is used for the topographical imaging of the sample.

A major drawback in all of these known methods is that before a sample can be examined, it must first be found. Thus, a region of the sample preparation which contains an object of interest must first be identified, and this can be difficult. Although attempts have been made, in connection with both STM and AFM, to associate these types of microscope with electron microscopes or conventional optical microscopes, for example, in order to take advantage of the wider range of scan of these more conventional instruments, searching for a suitable specimen can still waste a very large amount of time, because STM, AFM and SNOM inherently have a small width of scan. SNOM also requires the mounted sample to be positively approached towards an aperture or asperity in the gold film. As also with STM and AFM techniques, the mounting or immobilising of the sample in SNOM can result in damage to the sample. At the same time it is not possible to bring the optical probe close enough to the sample to be at an optimum distance from it without risking untoward close contact,or even impact, between the sample and the probe. Even so, the distance between the sample and the aperture has to be very closely controlled, and as with the other techniques, the equipment involved is somewhat complex and expensive.

Another disadvantage found with the current techniques is that, whereas resolution normal to the plane of the aperture in the instrument element defining the site at which examination of the sample is to take place (vertical resolution) is high, lateral resolution is much

lower. In the case of STM and AFM instruments, it is not possible to produce a probe tip narrow enough to produce lateral resolution comparable to the high degree of vertical resolution which is possible. This drawback is more marked in the case of the SNOM, and the smallest probe diameter is of the order of 30 nm. A further disadvantage is that interaction between the sample particles and the radiation scattered at the aperture may of necessity be relatively poor. Furthermore the SNOM is not believed to have been shown capable of analysing particles in solution.

There is in existence a device known as a Coulter (Trade Mark) counter for examining microscopic particles such as biological cells. The cells are passed, in suspension, through a hole connecting two bodies of a solution of a salt in which the particles are in suspension, and measuring the change in capacitance resulting from passage of the cells through the hole. However, the principle of the Coulter counter is not applicable on the small scale to which the present invention is directed: the smallest holes in a Coulter counter are of the order of 10 μm, whereas with the present invention the apertures are smaller than this by a factor of some 10^{-3}, and the physics involved are essentially those of quantum mechanics at this level.

Another method of detecting individual cells (1 - 5 μm and larger) is that of conventional flow cytometry, in which cells are hydrodynamically caused to pass through an optical scattering volume (laser beam) for analysis. As with other conventional optical systems, the resolution available with flow cytometry is limited by diffraction effects, which limit the degree of beam focussing that can be obtained.

According to one aspect of the invention, a method of examining individual objects (as defined above), includes the steps of:

(i) bringing a said sample into proximity with an instrument element comprising a substrate overlaid with at least one thin film layer of a material which is electrically conductive and/or at least partly optically opaque, the film layer having a discontinuity in a known or identifiable location;

(ii) applying energy to the instrument element to cause detectable radiation at the discontinuity;

(iii) causing relative movement between the sample and the discontinuity so as to bring them into intimate association with each other; and

(iv) continuing to apply the said energy while detecting the resulting changes in the radiation at the discontinuity.

Where the discontinuity is an aperture formed through the film layer, the "intimate association" referred to above consists in the presence of the sample, or part of the sample, in the aperture itself. Accordingly, samples suitable for examination with this arrangement will generally consist of particles and other bodies which can be brought into the aperture. The aperture is preferably larger than at least one expected dimension of the sample, but of a similar order of magnitude. However, the arrangement can also be used for study of the interaction between two samples, one of which may for example be partly in the aperture and the other one close to it.

If the discontinuity is an asperity projecting from the

film layer, the sample may or may not be of such a configuration that it could be mounted in or pass through an aperture. Use of asperities is especially suitable where the sample consists of a membrane or analogous structure. The "intimate association" can take any suitable form, depending on requirements: the sample may for example be in actual contact with the asperity, or almost in contact with it. In one type of practical embodiment, the portion of the film layer bearing the asperity can be coated with a sample membrane, with other samples, of any kind, then being brought into intimate contact with the latter at the asperity so that these samples can be analysed in terms of their interaction with the membrane by detection of the changes in radiation emanating from the asperity in the presence of such a sample.

In general, the instrument element with its discontinuity is preferably, though not necessarily, arranged in a fixed position, so that the relative movement between sample and discontinuity preferably consists in conveying or attracting the sample towards the discontinuity and into the appropriate intimate association with it, using electrophoresis or any other suitable means.

Preferably, the method also includes the further step of applying an energy field or fields to the discontinuity in such a way as to modulate or modify the behaviour and/or the structure of the sample. This additional or modulating energy may or may not be of the same kind as the basic energy which is applied to cause the detectable radiation from the discontinuity in the first place. This basic applied energy may be electrical energy or electromagnetic energy such as light. In the latter case, the aperture or asperity acts as a short optical

antenna.

The invention, in a second aspect, is directed to apparatus for examining individual objects (as defined above), including an instrument element in the form of a substrate overlaid with at least one thin film layer of a material which is electrically conductive and/or at least partly optically opaque, the film layer having a discontinuity in a known or identifiable location; means for causing relative movement between a sample and the discontinuity and for bringing them into intimate association with each other; and means for applying energy to the instrument element so as to cause detectable radiation at the discontinuity.

Considering the case where the discontinuity is an aperture, it is an important feature of the invention that the sample is actually brought into close proximity to, or preferably actually into the interior of the aperture itself. Thus, instead of examination of a sample being possible only when it lies in the near field region outside an aperture, into which radiation is scattered from the latter, as in conventional SNOM practice, the sample can actually be brought into the intimate association, discussed above, with the aperture as well as the near field. The invention thus enables advantage to be taken of tunnelling effects in addition to the near field optical effects which characterise known methods based on scanning. The net result is that the sensitivity of the apparatus is greatly increased, since the radiation field within the aperture, of a similar order of physical size to the particle, will have a profound effect on the behaviour of the radiation, and therefore a substantially increased effect on the radiation which "leaks" from the aperture. These changes

can be quite readily detected by conventional means. Similar considerations apply where the discontinuity is an asperity.

In one class of apparatus according to the invention, the instrument element has two electrodes applied over the substrate with a said aperture extending between them, the applied energy being electrical energy such as to set up a field or tunnelling current in the aperture. When a sample particle is in the aperture, this field current passes in contact with the particle itself, and the electrostatic field in the aperture becomes modified in accordance with the dielectric properties of those parts of the particle. The dielectric properties of the particle are thus used as the basis of measurement in this application of the invention. The effect is that the contact between the two electrodes is perturbed by the sample, and the resulting effects on the current can be accurately measured and analysed to give information about the particle. Perturbation of the particle by the field may also be detected optically.

In alternative embodiments, in which light is used as the source of energy, the film layer is then made at least partly opaque to light, the substrate being able, either by being porous or otherwise, to transmit light, so that light applied through the substrate leaks from it through the aperture in the film layer. In this case, as indicated above, the presence of the particle within the aperture itself will cause a considerably more marked disturbance of the light leaking from the aperture. Indeed, in this case conventional optical equipment will in most cases be sufficient to detect the changes in the visible light caused by the presence of the particle in the aperture.

The invention is particularly well adapted for the examination of samples in solution. An electrical potential is applied across the instrument aperture so that the solution, containing the sample in free solution in a solvent, migrates towards it, for example by electrophoresis, and particles or other samples in solution can thus themselves migrate to the location of the aperture and so into the latter. For this purpose, the substrate is porous and its electrical conductivity is low enough, i.e. it is a sufficiently good insulator, for the aperture and porous channel to act as a preferred leakage path.

The invention eliminates the need to scan a surface over which sample objects are attached, firstly in order to find a sample for examination in the first place, and secondly to examine the sample once it has been found. Instead, the sample is deliberately directed into a single predetermined location, where it is then examined. It follows that a plural number of samples can be simultaneously examined, using their separate interactions with a corresponding number of apertures and/or asperities.

Where this predetermined location consists of an aperture in or through the instrument element itself, the sample is examined by observing the interaction between the applied energy and the sample inside the aperture (or that part of it that is inside the aperture as the particle electrophoreses through the latter). The aperture is preferably of known dimensions, so that the detection volume (i.e. the volume of the aperture) is highly specific and suitable for the size and associated field of a sample passing through it. This results in a substantial reduction in unwanted "noise", and hence a

very significant improvement in signal-to-noise ratio, resulting in turn in a substantial improvement in resolution and accuracy of the usable output signals. These improvements are also evident where the discontinuity is an asperity. The invention also offers other advantages, some of which have already been mentioned above.

The invention is intended primarily for use on samples in which the interaction between the sample and the applied energy, e.g. light or electrical energy, is governed by quantum mechanics. It should however be understood that the invention is also applicable to the examination of microscopic objects large enough for more conventional laws to be applied. Many viruses for example, larger than about 30 nm in size, fall into this category.

A few examples of the application of the invention will now be described, by way of example only, with reference to the accompanying drawings, all of which are highly diagrammatic and not to scale. In the drawings:

Figure 1 shows part of an apparatus according to the invention for examining particles of macromolecular size by observing the disturbance caused by the particle on an electric field, using the dielectric properties of the particle itself as the basis of measurement;

Figure 2 is an enlargement of part of Figure 1, showing a particle in an aperture formed through an "instrument element";

Figure 3 shows another apparatus working on a similar principle to that of Figures 1 and 2, but modified to enable an elongate particle to be examined bit by bit as it passes through the aperture;

Figure 4 is a view as seen from one side of Figure 3, showing part of the instrument element in cross section on a larger scale than Figure 3;

Figure 5 shows in cross section part of the instrument element of another form of apparatus according to the invention, in which particles are caused to migrate through the instrument element at a known location, and are examined by observing the interaction between them and applied light;

Figure 6 is a cross section through part of the instrument element in yet another embodiment in which light is used, but in which the particle is held stationary in the aperture;

Figure 7 illustrates one use of the invention where the discontinuity is an asperity acting as a short optical antenna;

Figure 8 illustrates the use of piezoelectric effects to produce an applied energy field;

Figure 9 is a cross section of part of an instrument element incorporating a convergent waveguide;

Figure 10 illustrates a use of the apparatus for studies at a liquid-liquid interface; and

Figure 11 illustrates a use of the apparatus in DNA sequencing analysis.

Referring to Figures 1 and 2, the apparatus has two receptacles 10 and 12 separated by an instrument element 14 comprising a generally flat substrate layer or membrane 16 of insulating material, with two electrodes 18 and 20 formed on its two opposed outer faces. The

rear electrode 18 is on the side of the membrane facing into the receptacle 10, and the front electrode 20 faces into the receptacle 12. Both electrodes in this example are layers of metallic gold with a thickness which may be as little as a few nanometres or as much as a few microns. The electrodes 18 and 20 are connected to a unidirectional or direct-current electrical supply 22, and to instrumentation, generally indicated at 24, for receiving and processing signals from the instrument element 14.

In a known location on the element 14, an aperture 26 is formed, by any known method, through the electrodes and the substrate layer 16. The diameter of the aperture 26 is of the same order of magnitude as a macromolecular particle, though it must be large enough for the kind of particles which are to be examined to pass through it, and therefore larger than at least one expected maximum dimension of the particle, e.g. its transverse width. Furthermore, the transverse width of the particle must in this context be regarded as comprising not only the expected physical width of the particle itself, but also that of the characteristic electrostatic field, or double layer, of the particle which inherently surrounds the body of the particle and which may typically have a total thickness of the order of 20 nm. In addition, as will be seen, the apparatus is intended for examination of sample particles in solution, so that there will be a solvation layer several namometres thick around the particle due to the effect of self-alignment of water molecules with the surface of the particle. The aperture is preferably large enough to allow a particle to pass through it without the walls of the aperture distorting either the solvation layer or the electrostatic double layer. However, some such distortion may for some purposes be

desired, in which case the aperture size can be chosen accordingly.

If desired, the instrument element 14 may have a number of apertures 26, all in known locations but of different sizes so that the apparatus can effectively handle a variety of different kinds of particles of different sizes.

The receptacle 10 contains a solution, typically an aqueous solution (though any suitable solvent, liquid or gaseous, can be used), with particles of the kind which are to be examined in free solution in the solvent. A voltage is applied between the two receptacles 10 and 12, such as to cause the solution to migrate electrophoretically from the former towards the latter through the hole 26. This voltage may be applied from the source 22, or from another unidirectional supply source, not shown.

It will of course be understood that close interaction between the particle and the aperture may be obtained by any other suitable, and well-known, technique using a selective physical or chemical motive force. Examples include osmosis, diffusion and centrifugation.

Because of the voltage applied between the electrodes 18 and 20 from the source 22, a circuit is completed between the two electrodes within the aperture 26. When there is no particle in the aperture 26, the pattern of leakage paths within the aperture adopted by the current (possibly a tunnelling current) will be governed only by the geometry of the aperture itself. However, when a particle, 28 in Figure 2, enters the aperture as indicated in phantom, the dielectric properties of the particle will significantly change the behaviour of the

leakage or tunnelling current. The particle is shown in full lines about halfway through the aperture. As it passes through the aperture, the tunnelling or leakage current will tend to change continuously until it reverts to normal once the particle has passed out of the aperture. In this connection, it is important to note that, because the size of the aperture 26 is of the same order of magnitude as that of the particle 28, the paths taken by the leakage current will intersect the particle itself or its immediate, unique and characteristic micro-environment specifically associated with it. In the present context this micro-environment can be regarded as part of the particle, so that the leakage current paths can be said in all cases actually to pass both through the particle and over its surface. The particle is thus, in effect, interacting directly and intimately with the electrical energy which is applied.

The changes in the electrical field within the aperture 26 are received by the instrumentation 24 in the form of output signals which can be analysed in any desired way to reveal features of the particle 28. In particular, since it is the electrical properties of the particle that are primarily responsible for these changes, and since any change in the relative positions of atomic components of a macromolecule is known to produce predictable changes in the dielectric properties of the latter, the positions of such molecular (or atomic) components can be detected by analysis of the output signals. The instrumentation 24 may consist of commercially available hardware and need not be described here. It should be noted here that sample particles can be specifically modified or labelled to allow analyses to be carried out to better effect.

The apparatus preferably also includes a frequency modulator 30 of any known kind, for superimposing frequency modulation on the d.c. supply. Preferably the modulator 30 is also capable of varying the modulation frequency, so that the input signal can be "tuned" to various values corresponding to the natural resonance frequencies of different parts of a particle, or of different particles, under examination. This effect can then be used to cause different parts to reveal their presence by resonating at their own characteristic frequencies. The effects of such resonance will of course appear in the output signals detected and analysed by the instrumentation 24. Such effects may also be detected optically. The fields can be used for changing the conformation and kinetics of intersection with the aperture or another particle in the aperture.

In the modified instrument element 40 shown in Figures 3 and 4, there are again two electrodes, 42 and 44. Here, however, both electrodes are applied on the same side of the substrate element 16, in this example the rear. The electrodes are separated by a narrow gap 46, and the aperture 26 is formed through the insulating substrate layer 16 only, opening into the gap 46. There is again a leakage or tunnelling current, indicated at 52, between the two electrodes, passing across the gap 46, so that a particle 48 passing from one side of the element 40 to the other will pass generally across the path of the tunnelling or leakage current, instead of generally along it as in Figure 2. Thus, in the case of an elongate particle 46, as shown in Figure 4, successive sections of the particle (defined in Figure 4 between phantom lines 50) will cause perturbations to take place in the tunnelling current 52 that are characteristic of the different sections 50, thus enabling the particle 48 to

be examined bit by bit.

In the embodiment of Figures 3 and 4, the length of the sample sections 50 which can be examined individually is determined by the thickness of the electrodes 42 and 44, which are therefore preferably made as thin as possible so that as large a number of sections 50 as possible may be identified and analysed. Typically the electrodes 42 and 44 have a thickness of about 1 nm, the substrate layer 16 being for example of the order of 50 nm thick or less. The gap 46, and the hole 26, may be formed by conventional electron beam writing or any other suitable known technique. Frequency modulation, as described above, may equally well be applied to the embodiment of Figures 3 and 4.

Analysis of the object (i.e. particle, molecule or structure, as discussed at the beginning of this description) in the aperture can be carried out using time-resolved optical techniques (fluorescence), or any other analytical method operating in the time domain, and in which the object is specifically modulated, optically or electrically, so as to be optically or electrically phase locked into any given detection principle.

The applied energy may be a form of electromagnetic radiation instead of electrical energy as in the preceding examples, and in Figures 5 to 7 this electromagnetic energy is light. In Figure 5, an instrument element 60 is placed between the receptacles 10 and 12, the former containing a solvent in which particles 62 to be examined are in free solution, as before. The element 60 comprises a porous, translucent, electrically insulating substrate membrane 64, on the front face of which an opaque film layer 66 is deposited by any suitable means. The layer 66, which may typically

be of metallic gold, may have any suitable thickness, typically of the order of 20 nm. At least one aperture 68 is formed through the film layer 66, and, as in the preceding examples, the solution is caused to migrate electrophoretically in the receptacle 10 to the instrument element 60, so that the particles 62 pass through the aperture defined by the hole 68 and by the porosity of the membrane 64 immediately behind it. This "aperture", or path through the element 60, is generally indicated in Figure 5 by the reference numeral 70. The translucent membrane 64, which is preferably transparent, is edge illuminated, for example by means of a laser 71, at an angle of incidence such that it acts as a waveguide, as indicated by the directional lines 72 which represent light paths within the membrane-substrate layer. Although the light following the paths 72 is generally constrained within the membrane 64, some of it will leak outwardly through the hole 68 to give near-field light leakage or scattering as indicated at 74. The light paths 72 are at grazing incidences only, so that light does not penetrate the film layer 66, except at the holes 68 which act as the scattering centres.

As a particle 62 approaches the aperture or porous path 70 under electromotive force, it will start to interact with the electromagnetic field associated with the aperture which acts as a short optical antenna (SOA) according to the well-known principles explained above. The effect of this is that the scattered light 74 will change in respect of at least one optical parameter, such as intensity, polarisation, phase, spectral content or fluorescence. This effect will increase as the particle approaches the hole 68, and be strongest when the particle is actually in that hole. The scattered radiation 74, modified by the particle, therefore

represents an output signal which can be received by any suitable optical receptor. In this example, the latter is a conventional microscope objective lens 76, typically of x40 magnification.

The optical signal from the objective lens 76 may then be processed and analysed by any suitable optical and/or electro-optical instrumentation, not shown.

It should be noted that this process again involves interaction between the light itself, i.e. the electromagnetic field associated with the aperture 68, acting as an SOA, and the electrostatic or dielectric characteristics of the particle. Accordingly, the porosity of the membrane 64, and, once again, the size of the hole 68,are chosen to take into account the electrostatic double layer surrounding the particle and forming, in electrostatic terms, part of the particle itself. The hole 68 is typically from 20 to 200 nm in diameter. We shall return later herein briefly to the significance of this double layer.

The opaque layer 66 may be made unsupported, being back-illuminated at suitable grazing incidence angles such that light does not pass directly through the hole 68 but only escapes from it by virtue of small aperture/asperity scattering effects. However, where the layer 66 is supported by a substrate 64, the latter may be of any suitable material which is porous to the macromolecule, for example hydrophilic or hydrophobic gel, compatible with the solvent in which the particles 62 are carried in free solution. Another possible material for the membrane is controlled-pore glass. The objective 76 is focussed on one particular selected hole 68, and the film layer 66 may be formed with no other perforations. It may however have a large number of perforations, and

Figure 5 shows more than one. Particles passing through any of these other perforations are of no account. Alternatively, of course, perforations of different sizes, each in a known location onto which the objective 76 can be focussed beforehand, may be provided for the examination of particles of different sizes in the same way as has been suggested above in connection with the electrical method described with reference to Figures 1 to 4.

Reference is now made to Figure 6, in which the instrument element, 90, does not define a path for a particle through the element itself. Here the aperture consists merely of the hole 68 formed in a film layer 66, generally similar to that described with reference to Figure 5 and mounted on a transparent waveguiding substrate layer 92, which need not be porous but may merely consist of optical glass. The substrate layer 92 is again edge illuminated at grazing incidence, so that the only light passing out through the hole is the leakage radiation 74. The glass substrate 90 is suitably treated by any conventional method for activating it, for example (where it is of glass) by an immobilisation technique such as silanisation such as to enable the samples, for example biological macromolecules, to be simply attached to the glass. Thus, with the substrate 92 of glass and the opaque layer 66 of gold, if the resulting element 90 is treated by a liquid or vapour phase silanisation technique, since gold reacts poorly to such immobilisation chemistries, the element 90 will be activated substantially only on the parts of the glass surface exposed within the hole or holes 68. The sample macromolecules, 96 in Figure 6, will therefore tend to become attached, by a silane immobilising ligand 98, within the holes themselves. Thus, as before, a signal

receptor such as the objective 76 in Figure 5 can be focussed on one particular hole 68, the particles adhering anywhere else on the element 90 being of no account. Because particles will preferentially attach themselves in holes 68, it is merely necessary to focus on to one of the latter, without the need for any scanning to locate a particle for examination. In this connection, it should be noted that even if some attachment of particles to the gold surface does occur, these will cause no interference since the useful output signals are generated only by structures associated with the hole 68.

When a particle is trapped in a specific location in this way, experiments can be carried out for a number of different purposes on the stationary particle at leisure. In particular, the arrangement shown in Figure 6 may be used for the detection and analysis of other bodies interacting with the sample or samples located in the aperture 68. It follows that a sample, such as a particle, in the aperture 68 can be augmented with another material, or composite, of suitably small size, which may interact with the sample in such a way as to cause changes to take place in its optical and/or electrical properties. These changes can then be detected and analysed.

Figure 7 illustrates another embodiment in which the effects of interaction can be studied. In this case the transparent substrate, 100, again has a thin gold film, 102, on one of its surfaces, but here the discontinuity is an asperity instead of an aperture. The asperity is a projection 104 on the outer side of the film 102, which is coated with a layer of a sample material, such as a biological membrane 106, with this layer covering the

asperity 104. Other samples, such as a membrane 108 or a particle 110, are brought by electrophoresis or otherwise into contact with, or very close proximity to, the membrane 106 in the region 112 where the latter overlies the asperity 104, such that in this region there is interaction between the transported sample 108 or 110 and the membrane 106.

Light is applied to the instrument element 100, 102, for example via the substrate 100 as already described in connection with Figures 5 and 6. The film 102 is thin enough to be only partly opaque to this light so that some light passes through it, the remainder of the light being reflected by the film. The asperity 104 acts as a short optical antenna, causing scattering of both transmitted and reflected light.

When interaction occurs between a sample 108 or 110 and the biological membrane 106, the resulting changes in the light emanating from the SOA asperity 104 can be detected. This may be done, for example, using a transmitted-light lens 114 on the same side of the instrument element as the asperity, or a reflected-light lens 116 on the other side of the element, since both the transmitted and reflected light will be affected by the interaction between the samples.

The configuration of the asperity 104, and its dimensions, can be chosen to suit the particular application for which it is to be used.

Another example, using the instrument element 90 of Figure 6, is illustrated in Figure 11. It should be noted that the arrangement seen in Figure 7 could, however, be used instead. In Figure 11, a polymerase molecule 120 is attached by a ligand 98 in the aperture

68 over the waveguide substrate 92. A single-stranded DNA molecule 122, with a primer sequence 124 base-paired on to it in a known manner to initiate polymerisation, is brought to the polymerase 120. The progress of the resulting polymerisation with nucleotide bases 124 and fluorescently-labelled bases 126, giving the duplex DNA structure 127, can then be observed and analysed due to the changes in the light emanating from the aperture 68, light being supplied to the substrate 92 as before.

A chemical or biochemical passivation or modification layer 128, of suitable composition, may be applied over the outer surface of the metal layer 66 in order to improve the specificity of binding of the target DNA molecule 122, and/or to reduce non-specific binding. It should be noted that such a layer 128 can be employed in all variants of the method or apparatus of this invention where these effects may be required.

The instrument element can be configured in a miniature waveguide form, e.g. as a slab or other waveguide, or in a fibre optic form. In the latter case it is, for example, capable of being used as a remote, multiplexable fibre optic sensor which can be incorporated, if required, into a network.

Figure 9 shows one example in which the substrate 130 of the instrument element, having a metal film layer 66 and an aperture 68 as before, is of glass so as to act as a monomode optical waveguide. A convergent surface profile 132 is formed on the rear face of the substrate 130 leading to the aperture 68. This profile acts as an efficient, tapered, local wavguide, enhancing containment of the incident light and concentrating the latter at the aperture 68 while reducing interfacial losses.

The convergent profile 132 terminates in a hole 134 aligned with the hole 68 in the layer 66 so that the aperture 68 itself comprises these two holes, which are preferably drilled by high-energy electron beam lithography after suitable masking (a hole-forming technique which can be used for any of the apertures in the various embodiments of the invention in which the discontinuity serving as the working site is an aperture).

The concentration of light at the aperture, together with the reduced losses, may produce flare effects involving sub-wavelength tunnelling effects which can enhance the observable changes that supply the information required when a sample is present at the aperture.

The profile 132 can be of any desired shape, e.g. spherical or conical, and may be formed by etching.

Another possible use of apparatus according to the invention is in the study of a stable liquid/liquid interface 140, Figure 10, or in the study of the behaviour of molecular objects at such an interface. Figure 10 shows two such molecules, 142, suspended at the interface within the aperture 68 of an instrument element 143 which (in this example) happens to be similar to that shown in Figure 9, but which need not have a tapered waveguide section. A first liquid solvent 144 is above the element 143, and a second liquid solvent 146 below it. These solvents will be chosen at least partly so that their physical characteristics permit the stable interface 140 to form in the aperture 68. They, and/or the samples, will also be chosen so that the latter are ormphiphilic.

Referring now to Figure 8, an applied energy field can be

created piezoelectrically. In Figure 8, the substrate 150 of the instrument element is of an optically-transparent piezoelectric material to which a pair of electrodes is fitted, the electrodes being connected through a piezoelectric resonator circuit or driver 152. Depending on the piezoelectric polarity, these electrodes are mounted so as to transmit impulses through the substrate 150 either transversely (electrodes 154) or longitudinally (electrodes 156 in phantom lines). In the former case the whole of the metal layer 66 may serve as an electrode.

With a sample 158 mounted in the aperture 68 in the layer 66, light 72 is transmitted through the substrate as described with reference to Figures 5 and 6. In addition, the driver 152 is energised so as to superimpose piezoelectric energy on the substrate, at a frequency which excites the sample at an acoustic (audio or ultrasonic) frequency and modulates the observed optical output. This produces the well-known phenomenon of Brillouin scattering, which depends on the interaction of the applied light and acoustic energy and also on the characteristics of the sample which determine the optical output. Data on such characteristics can in this way be obtained.

The electrostatic double layer of a macromolecular particle has been mentioned above. This is part of its characteristic electrostatic field. In the case of certain biological macromolecules, small differences in primary structure lead to significant differences in their function. Current theories on protein, enzyme and other macromolecule structure and function suggest that secondary and tertiary structure is responsible for generating large electrostatic fields, which may extend

to the equivalent of several diameters of the molecule itself. This is true for example in the case of some proteins, especially enzymes. Theoretical studies of these electrostatic fields have indicated a role in the capture of specific substrate molecules at relatively great distances by an enzyme macromolecule. The fields are generally toroidal in shape, and it has been shown that they have their poles centred on the active site of the enzyme, and that they act not only to facilitate interaction between the active site and the substrate molecule, but also align the substrates on their approach to the active sites. The structure and shape of these fields are accordingly likely to be highly characteristic of a specific enzyme, and the present invention provides a useful and versatile method of studying and exploiting these characteristic electrostatic fields in detail.

It should be noted that the invention is not confined to the case where the samples are in solution. Where they are, however, the solvent need not be water or even liquid, but the solution may take any form known to physical chemistry in which the sample particles can be caused to migrate to the examination site, either electrophoretically or under any other selective physical or chemical motive force. One example of such a solution is a gel.

Where the energy used for observation is light, i.e. in optical embodiments, a number of different optical parameters may be studied at the aperture or asperity, notably Brillouin scattering (already mentioned), intensity, fluorescence, polarisation and absorption. The intensity of light scattered at the aperture or asperity (the discontinuity) is modulated by the mass or dielectric of analyte samples in the vicinity of the

discontinuity if the latter acts as a scatterer. As to fluorescence, aspects of this that are available for study include intensity of fluorescence, shifts in excitation or emission wavelengths or profiles, decay patterns of fluorescence, and Raman scattering. Fluorescence may be observed by conventional spectroscopy using the apparatus of the invention.

Where a sample is optically active, changes in their polarisation may be detected, including depolarisation.

Absorption of light by a sample can manifast itself in the form of changes in intensity of the incident light, and can cause local heating around the sample (e.g. a molecule), so changing the local optical environment, which may then be analysed, for example by a photothermal technique. Again, absorption may be studied using conventional spectroscopy using the apparatus of the invention.

The thin film, which in the examples given above is of gold, can in practice be of any material that is suitable in terms of electrical conductivity and/or opacity, and which may or may not be a noble metal.

Besides the embodiments of the method and apparatus described above, the invention can be employed in a variety of other configurations and for a variety of other purposes, involving simple examination and analysis of samples; modification of the behaviour and structure of individual samples with examination and analysis of such modifications and/or their effects; and study of the interaction between a plurality of samples and/or between one or more samples and their environment.

Whatever embodiment of the invention is employed, and for

whatever purpose, it will be seen that, since the need for scanning to find a sample is avoided, and since consequently the examination site can be predetermined in advance, it is also possible to calibrate that site in conjunction with the parts of the apparatus that receive the signals and analyse them to give information about the sample. This will tend greatly to decrease the possibility of inadvertent error, such as may occur when the location of the site is randomly determined only by the fact that a sample happens to have settled there.

It should however be noted that use of the method and apparatus of the present invention does not necessarily preclude its combination for certain purposes with known forms of equipment. For example, the apparatus of Figure 6 may be incorporated in a scanning tunnelling microscope, so that the STM probe can be used to pick up a molecule and place it on the sample macromolecule already attached in the hole 68, so that the resulting effects can be observed. The probe releases the molecule being carried to the site simply by changing the voltage to the probe. Similarly of course the added molecule may be removed.

CLAIMS

1. A method of examining samples comprising individual objects (28, 48, 62, 96, 158, 142, 127) of a size in or smaller than the same general order of magnitude as macromolecules or their aggregates, characterised by the steps of:

(i) bringing a said sample into proximity with an instrument element (14, 40, 60, 90, 143) comprising a substrate (16, 64, 92, 100, 130, 150) overlaid with at least one thin film layer (20, 18, 66, 102) of a material which is electrically conductive and/or at least partly optically opaque, the film layer having a discontinuity (26, 68, 104) in a known or identifiable location;

(ii) applying energy (22, 72) to the instrument element to cause detectable radiation at the discontinuity;

(iii) causing relative movement between the sample and the discontinuity so as to bring them into intimate association with each other; and

(iv) continuing to apply the said energy while detecting the resulting changes in the radiation at the discontinuity.

2. A method according to Claim 1 wherein, the discontinuity being an aperture (26, 68) through at least the film layer (20, 66, 102), step (iii) comprises bringing the sample (28, 48, 62, 96, 142) into the aperture.

3. A method according to Claim 2 wherein, the discontinuity being an aperture (26, 70) extending

through the instrument element (14, 40) to define a passage therethrough, step (iii) comprises passing the sample (48, 62) through the passage.

4. A method according to Claim 1 wherein, the discontinuity being an asperity (104) on the film layer (102), step (iii) comprises bringing the sample (110) into contact, or almost into contact, with the asperity.

5. A method according to Claim 3, wherein step (iv) comprises detecting said changes repeatedly as the sample (48) passes through the passage (26), so as to produce data relating to successive parts (50) of the sample.

6. A method according to Claim 2 or Claim 4, including at the end of step (iii) the further step of substantially immobilising the sample (96, 110, 142) in its said association with the discontinuity (68, 104, 68) while step (iv) is performed.

7. A method according to any one of the preceding Claims, wherein step (ii) comprises applying an electrical voltage to the instrument element (14).

8. A method according to any one of Claims 1 to 7, wherein step (ii) comprises applying light (72) to the instrument element (60, 92, 130).

9. A method according to any one of the preceding Claims, including applying to the sample (28 etc.) and/or the instrument element (14 etc.), at least while step (iv) is being performed, an energy field such as to modify or modulate the behaviour and/or structure of the sample.

10. A method according to Claim 9, wherein application of the said energy field comprises modulating the energy

applied in step (ii).

11. A method according to Claim 9 or Claim 10, wherein the said energy field is applied as a different form of energy from that applied in step (ii).

12. A method according to Claim 11 when dependent on Claim 8, wherein the said different form of energy is in the form of acoustic or ultrasonic excitation of the sample, step (iv) comprising detecting changes in light (72) emitted from the site of the sample (158) resulting from the said excitation.

13. A method according to Claim 11 or Claim 12, wherein the step of applying a said energy field comprises causing the substrate (150) to vibrate piezoelectrically whereby to produce excitation of the sample (158).

14. Apparatus for use in the examination of samples comprising individual objects (28, 48, 62, 96, 158, 142, 127) of a size in or smaller than the same general order of magnitude as macromolecules or their aggregates, characterised by:

an instrument element (14, 40, 60, 90, 143) in the form of a substrate (16, 64, 92, 100, 130, 150) overlaid with at least one thin film layer (20, 18, 66, 102) of a material which is electrically conductive and/or at least partly optically opaque, the film layer having a discontinuity (26, 68, 104) in a known or identifiable location; means (22) for causing relative movement between a sample and the discontinuity and for bringing them into intimate association with each other; and means (22, 71) for applying energy (72) to the instrument element so as to cause detectable radiation at the discontinuity.

15. Apparatus according to Claim 14, wherein the discontinuity comprises an aperture (26, 68) through at least the film layer (20, 66, 102).

16. Apparatus according to Claim 15, wherein the discontinuity comprises an aperture (26, 70) extending through the instrument element (14, 40) to define a passage therethrough.

17. Apparatus according to Claim 14, wherein the discontinuity is an asperity (104) on the film layer (102).

18. Apparatus according to any one of Claims 14 to 17, having at least one said film layer (18, 20), each of a said material, overlaid on each side of the substrate (16).

19. Apparatus according to any one of Claims 14 to 18, wherein the substrate (92, 100, 130, 150) is transmissive to the applied energy (72), so as to convey the latter to the discontinuity (68, 104).

20. Apparatus according to Claim 19, wherein the substrate (92, 100, 130, 150) is translucent, the energy-applying means comprising a light source (71).

21. Apparatus according to Claim 19 or Claim 20, wherein the substrate (92, 100, 130, 150) is configured as a waveguide for directing the applied energy to the discontinuity (68, 104).

22. Apparatus according to Claim 21, wherein the substrate (130) has at least one tapered portion (132) convergent towards the discontinuity (68) for concentrating the applied energy (72) at the latter.

23. Apparatus according to any one of Claims 14 to 22, wherein the said energy-applying means (22) are arranged to apply energy in such a way as also to cause the relative movement that brings the sample (28, 48) and the discontinuity (26) into intimate association.

24. Apparatus according to any one of Claims 14 to 23, further including modifying means (30, 152, 154, 156) associated with the instrument element (14, 66, 150), for applying to a sample (28, 158) at the discontinuity an energy field such as to modify or modulate the behaviour and/or structure of the sample.

25. Apparatus according to Claim 24, wherein the modifying means comprise modulating means (30) for modulating the energy that causes the said detectable radiation.

26. Apparatus according to Claim 24 or Claim 25, wherein the modifying means comprise second energy-applying means (152, 154, 156) for imposing on the instrument element a said field of a different form of energy from that (72) which causes the detectable radiation.

27. Apparatus according to any one of Claims 14 to 26, wherein the substrate (150) is of a piezoelectric material, the apparatus including means (152) for exciting the substrate piezoelectrically.

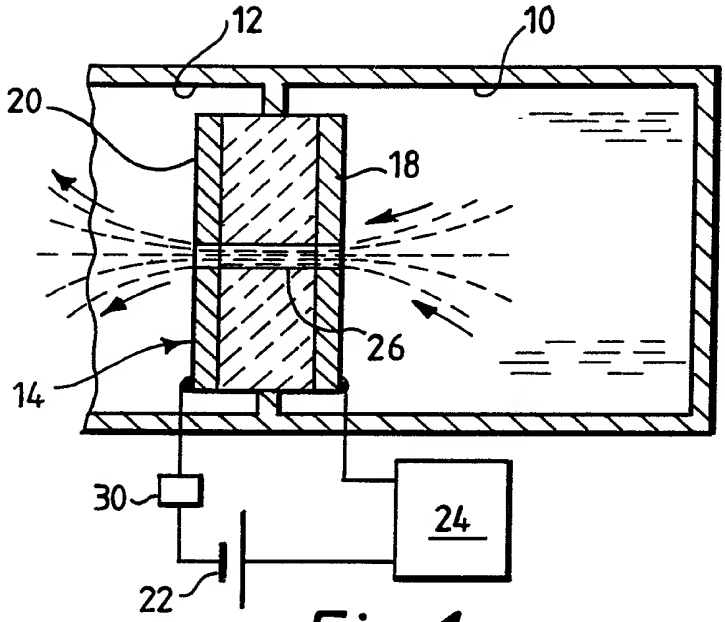

Fig.1.

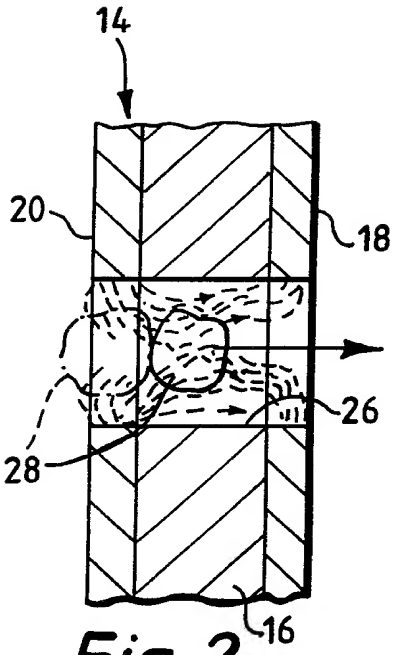

Fig.2.

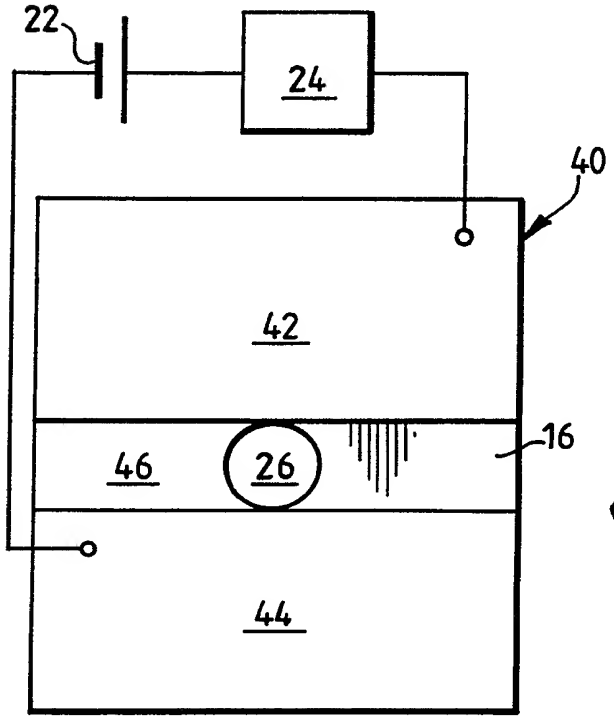

Fig.3.V

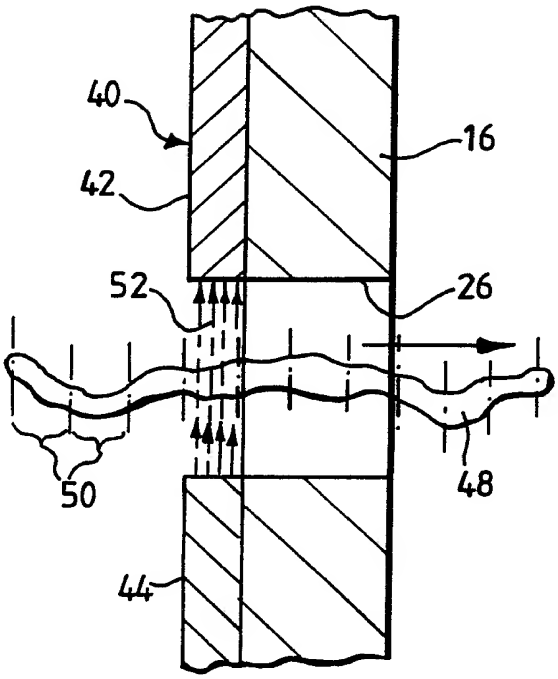

Fig.4.

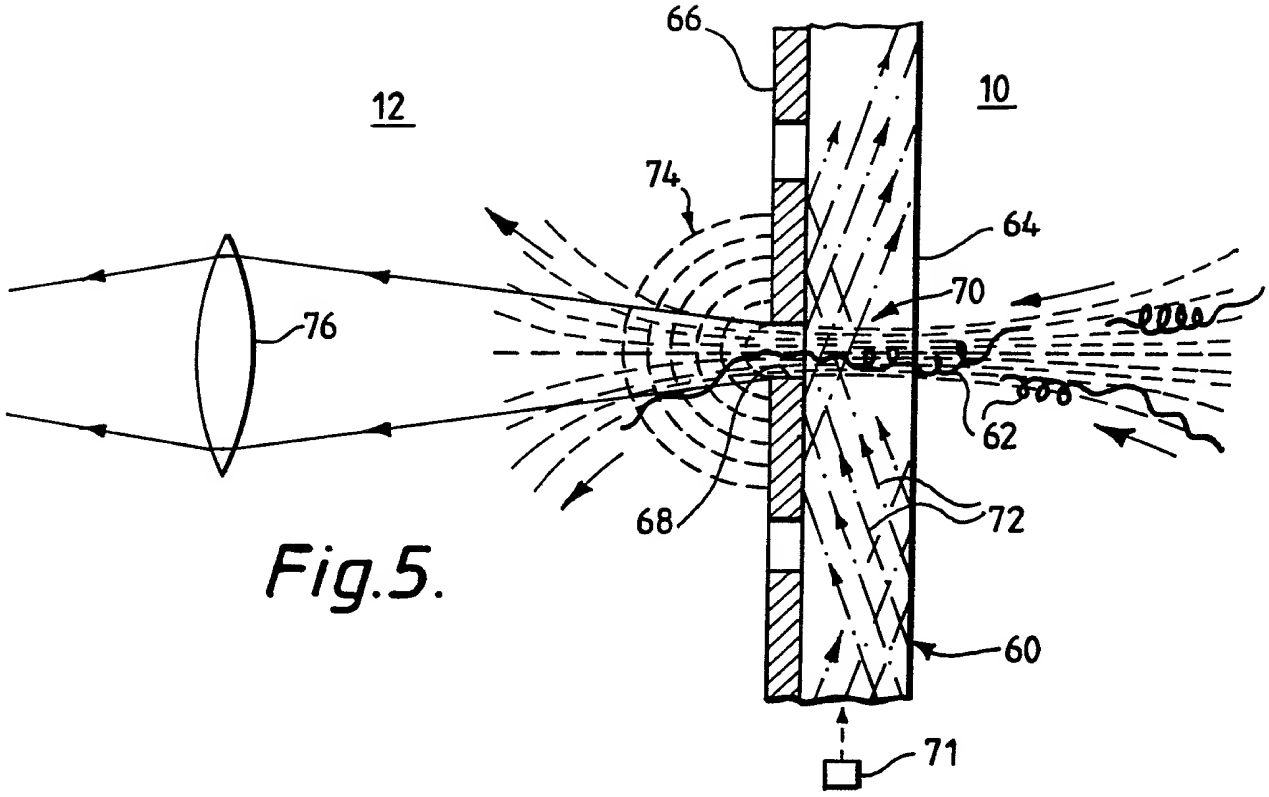

Fig.5.

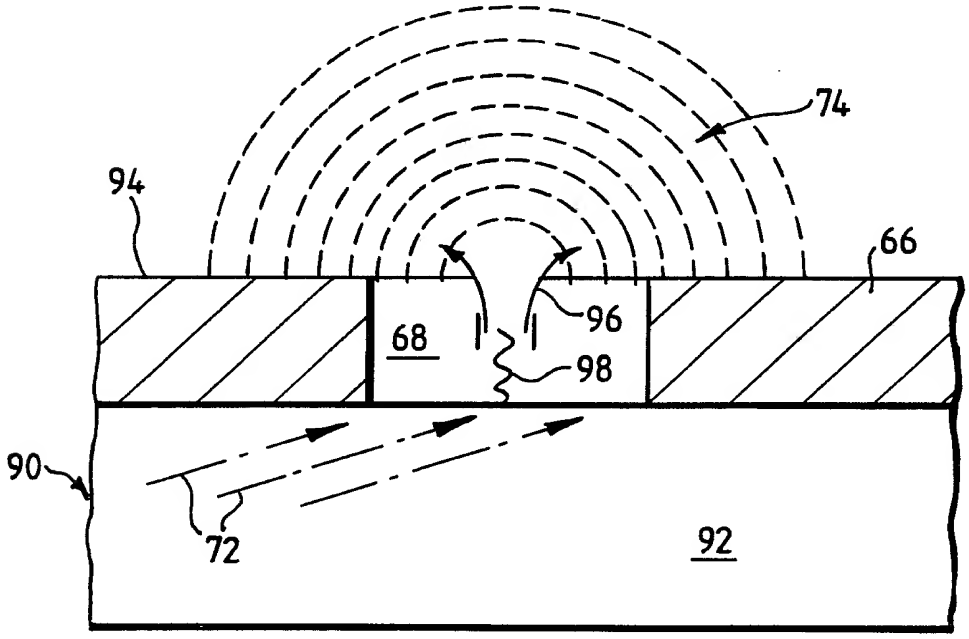

Fig.6.

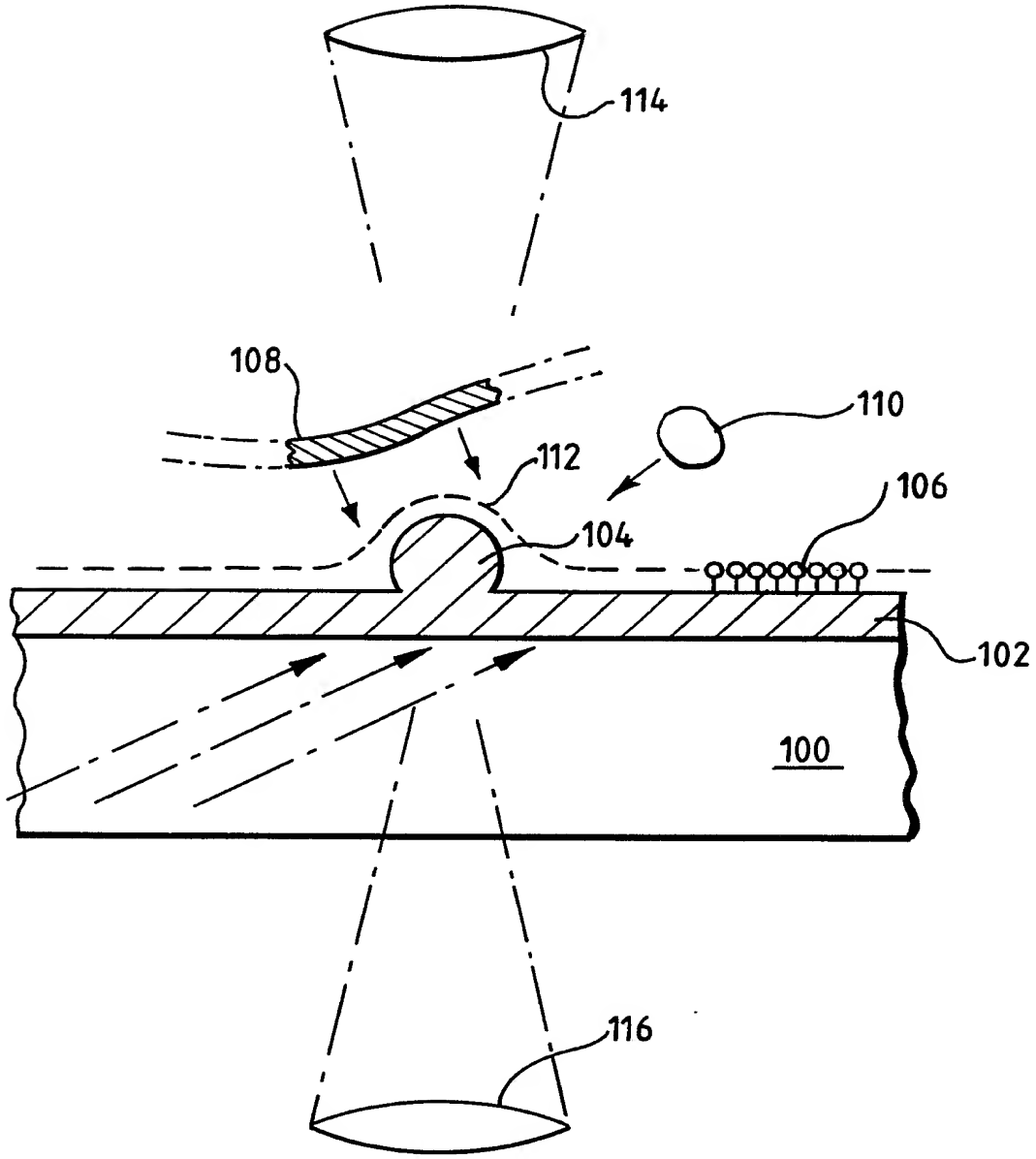

Fig. 7.

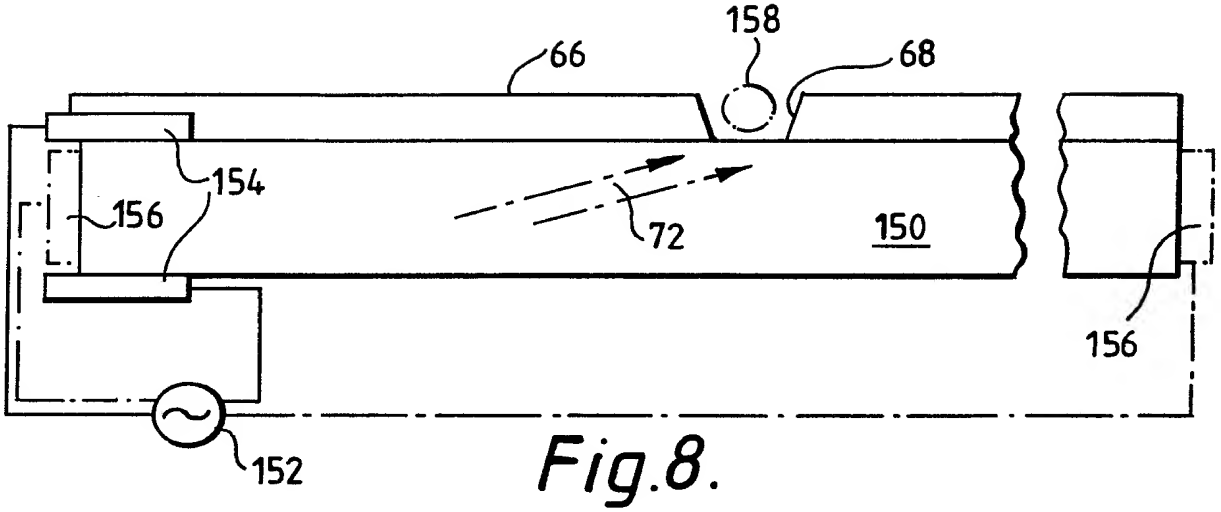

Fig.8.

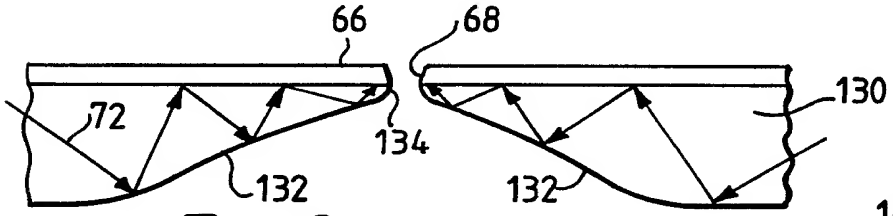

Fig.9.

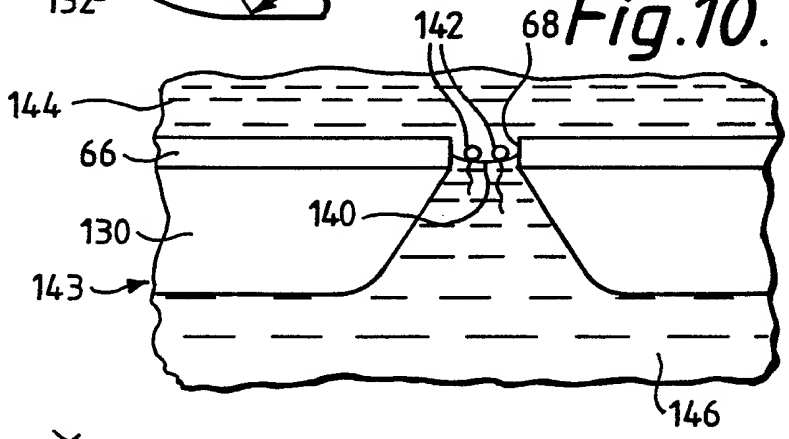

Fig.10.

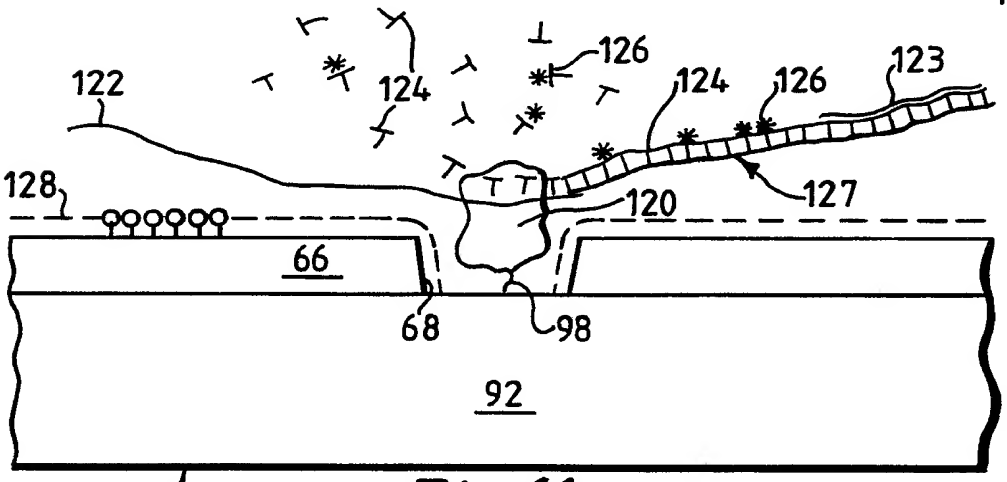

Fig.11.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 90/01407

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: G 02 B 21/00, G 01 N 21/62

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	G 01 N 21/17, G 02 B 21/00, G 01 N 21/62

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	EP, A, 0308537 (I.B.M. CORP.) 29 March 1989 see columns 6-12; figures 1-4	1,4,8,14, 17-21,23
X	IBM Journal of Research Development, volume 30, no. 5, September 1986, (Armonk, New York, US) U. Dürig et al.: "Near-field optical scanning microscopy with tunnel-distance regulation", pages 478-483 see pages 479-480, paragraph 2: "NFOS design"	1,2,14
A	EP, A, 0296262 (IBM CORP.) 28 December 1988 see column 3; column 4, lines 1-51	1,14

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* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
20th December 1990	21. 01. 91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. PEIS M. Peis

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	Journal of Vacuum Science & Technology B, volume 3, no. 1, January-February 1985, American Vacuum Society, (New York, US) U. Ch. Fischer: " Optical characteristics of 0.1 mum circular apertures in a metal film as light sources for scanning ultramicroscopy", pages 386-390 see pages 386-388, paragraphs II - IV ---	1,2
A	Applied Physics Letters, volume 52, No 4, 25 January 1988, American Institute of Physics, (New York, NY. US) U. Ch. Fischer: "Near-field optical scanning microscopy in reflection", pages 249-251 see the whole article ----------	1,2

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9001407 SA 40248

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 15/01/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0308537	29-03-89	JP-A- 1102302	20-04-89
EP-A- 0296262	28-12-88	JP-A- 1012201	17-01-89
		US-A- 4918309	17-04-90